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# Assessment of the potential risks associated with the proposed use of composted waste from the production of bacitracin as a soil additive

Norwegian Scientific Committee for Food Safety

Panel on Biological Hazards

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# Contents

# I- Summary

Bacitracin is a hexapeptide antibiotic, with a substituted thiazolidine nucleus, produced by some strains of *B. licheniformis*. It is mainly active against Gram-positive bacteria, although many differences in susceptibility exist among the bacterial species.
Alpharma A.S. Norway has produced bacitracin for use in human medicine since 1954. Until 1998, the fermentation waste from the production of bacitracin was added to animal feed in some European countries, including Norway, to promote growth of pigs and domestic fowl. In 1998, fermentation waste containing bacitracin as a food additive was banned by the EU to reduce the risk of developing bacitracin-resistant bacteria in animals, and the subsequent possible transfer of such bacteria to humans via the food chain. Use of fermentation waste containing bacitracin as a feed additive has not been officially banned in Norway, but it is no longer used for this purpose. Alpharma is therefore actively seeking alternative uses for their production waste. As the waste material is rich in nutrients, the company proposes that it could be developed as a soil additive by fermenting it with chipped bark and lime.
The Norwegian Food Safety Authority (Mattilsynet) commissioned the Panel on Biological Hazards of the Norwegian Scientific Committee for Food Safety (Vitenskapskomitéen for mattrygghet) to develop a risk assessment regarding the use of composted waste material from Alpharma's production of bacitracin, as a soil additive. In response, an *ad hoc* Working Group of experts was appointed with the mandate to draft a risk assessment which should include the following elements: assessment of risk to human health and/or the environment in relation to residual content of bacitracin in the finished soil additive product and assessment of the risk in relation to dissemination of the production strain and antimicrobial resistance genes.
The Panel on Biological Hazards concludes that the risks to human health and the environment posed by residual bacitracin present in the finished product are minimal. Furthermore, as *Bacillus licheniformis* is considered essentially non-pathogenic, occurring rarely as an opportunistic pathogen, the risk posed by this bacterium to human health or the environment is very low. It is reasonable to assume that during the early composting process horizontal transfer of bacitracin and erythromycin resistance genes, from the *B. licheniformis* producer strain to environmental bacteria, will exceed background levels. However, this is considered to represent a low risk to human health and the environment.

# II- Background

The antibiotic bacitracin, produced by the bacterium *Bacillus licheniformis*, is a mixture of several high molecular weight polypeptides (bacitracin A, B, and C and several minor components). When bacitracin was introduced into human medicine for clinical use, it was found to be nephrotoxic, and preparations for systemic use were soon withdrawn. Currently, bacitracin is widely employed as a topical remedy for wounds or ophthalmic ointment (Phillips 1999).
In this document the use of bacitracin as a feed additive refers to the use of the waste produced from bacitracin fermentation. Alpharma AS has produced bacitracin for use in human medicine since 1954. Until 1998, the fermentation waste from bacitracin production was added to animal feed in Norway and some other European countries to promote the growth of pigs and poultry. This practice is known to result in improved weight gain, and consequently less food is needed to bring the animals to their required slaughter weight. In 1998, bacitracin as a feed additive was banned by the EU to reduce the risk of developing bacitracin-resistant bacteria in animals, and subsequently possible transfer of such bacteria to humans via the food chain. Use of bacitracin as a feed additive has not been officially banned in Norway (FOR 2005-04-12 nr. 12: Forskrift om tilsetningsstoffer til bruk i fôrvarer), but the industry implemented a self-imposed ban on all growth promoters in 1995, following the ban of avoparcin as a feed additive. Therefore bacitracin has not been used as a feed additive during the last ten years. Alpharma is therefore seeking alternative uses for their production waste. As the waste material is rich in nutrients, the company proposes that it could be developed as a soil additive by fermenting it with chipped bark and lime. If approved by the Norwegian Food Safety Authority (Mattilsynet), the resulting compost, estimated to be about 1700 metric tons per year, will be sold on the open market.

# III- Legislation

According to the Regulation on Fertilizer Materials of Organic Origin[1]:
§9
Import of, or trade with, products that do not fulfil the quality requirements laid down in this regulation is prohibited. The Norwegian Food Safety Authority can ban products that entail an environmental risk or can harm or affect the health of plants, animals or human beings.
§10.2

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§9

Det er forbudt å importere eller omsette produkter som ikke tilfredsstiller de kvalitetskrav som går fram av denne forskrift. Mattilsynet kan i alle tilfelle forby produkter som kan medføre miljørisiko ved bruk, eller som kan skade eller redusere menneskers, dyrs eller planters helse.

§10.2

Den som produserer eller omsetter produkter etter denne forskrift skal vise aktsomhet og treffe rimelige tiltak for å begrense og forebygge at produktet inneholder organiske miljøgifter, plantevernmidler, antibiotika/kjemoterapeutika eller andre miljøfremmede organiske stoffer i mengder som kan medføre skade på helse eller miljø ved bruk.

The producer or traders must show precaution and lay down measures to prevent the product containing environmentally hazardous organic substances, pesticides, antibiotics/chemotherapeutic substances or other organic pollutants in amounts that pose risks to human health or to the environment when used.

# IV- Terms of reference

The Norwegian Scientific Committee for Food Safety (Vitenskapskomitéen for mattrygghet) has been commissioned by the Norwegian Food Safety Authority (Mattilsynet) to assess the risks of using composted waste material from Alpharma's production of bacitracin as a soil additive[2]:
Three questions, under two headings, of particular concern have been raised by the Norwegian Food Safety Authority:

**1- Assessment of risk in relation to residual content of bacitracin in the finished product.**
Will the residual content of bacitracin in the proposed compost pose any risk to human health or the environment?

**2- Assessment of risk in relation to dissemination of the production strain**
a- Assess the risks of disseminating *Bacillus lichenformis* 20443 to the environment, and whether such dissemination could pose a threat to human health or the environment.

b- Assess the risks of dissemination of antimicrobial resistance genes from the compost.

# V- Hazard identification

Production, handling, and application of composted waste material from the production of bacitracin may involve several hazards, as follows:

- Dissemination of bacitracin
- Dissemination and proliferation of the *B. licheniformis* production strain
- Dissemination and transfer of bacitracin, sulfonamide and erythromycin resistance genes to animal and human pathogens

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**2**

**Vurdering av risiko knyttet til restinnhold av bacitracin**
Vi ber om en vurdering av om inneholdet av bacitracin i kompostenutgjør noen helse- eller miljø risiko.

**Vurdering av risiko knyttet til spredning av produksjonsstammen**
Vi ber om en vurdering av om spredning av *Bacillus licheniformis* kan spres fra komposten, og om dette eventuelt utgjør noen risiko for helse eller miljø,
Vi ber om en vurdering av risiko knyttet til spredning av gen materiale som koder for antibiotikaresistens fra denne komposten.

# VI- Hazard characterization

## VI-I- *Bacillus licheniformis*

The *B. licheniformis* 20443 strain used for bacitracin production was derived from *B. licheniformis* ATCC 10716 by conventional mutagenesis and is not a product of recombinant DNA technology. The parent strain was originally isolated from soil and produced bacitracin at a lower rate. The *B. licheniformis* 20443 strain used by Alpharma is reported to be resistant to bacitracin, sulfonamides and erythromycin, although the method used for susceptibility testing is not according to standardized methodology. According to Alpharma AS, there is no information regarding the association between phenotypical resistance against sulfonamides and the presence of resistance genes. However, resistance against bacitracin and erthromycin is associated with the *bcr* gene cassette and the *ermD*gene, respectively (reported during meeting with representatives from Alpharma; 01.02.06).

### VI-I-i- Hazards to humans, animals, plants and environment

*B. licheniformis* appears to have a very low degree of virulence. In general, it does not produce significant quantities of extracellular enzymes or other factors likely to enable it to cause infection. There are some reports in the literature of human infections with *B. licheniformis*, however, these have all occurred in immunosuppressed individuals. In order for infection with *B. licheniformis* to occur it is probable that either the infectious dose must be very high, or the immune status of the host must be severely reduced (Edberg 1992). However, while the likelihood of *B. licheniformis* infection in humans is low, it is not nonexistent. Toxin-producing strains of *B. licheniformis* have been obtained from foods involved in food-poisoning incidents, from raw milk, from industrially-produced baby food (Salkinoja-Salonen *et al.* 1999), cooked meats, and vegetables (Rosenkvist and Hansen 1995;Tatzel *et al.* 1994).

There are numerous reports in the literature which associate *B. licheniformis* with livestock abortions (McClung 1992). It has been reported that *B. licheniformis* can be associated with abortions in cattle, sheep and swine (Kirkbride *et al.* 1986;Logan 1988). In Norway, two cases of abortion caused by *B. licheniformis* in dairy cattle were reported in 2003 (Bergsjø 2003). It is believed that hay and silage of poor quality may be predisposing factors.

No reports in the literature indicate that *B. licheniformis* is a plant pathogen. On the contrary, it has been reported that *B. licheniformis* in composting material promotes the growth of plants.

As *B. licheniformis* has only infrequently been associated with infections in humans, antimicrobial susceptibility testing of *B. licheniformis* strains has gained little attention. The species is reported to be intrinsically intermediately resistant to penicillin (Coonrod *et al.* 1971;Weber *et al.* 1988). Data regarding susceptibility of *B. licheniformis* to other antimicrobial agents is sparse.

In the opinion of the Scientific Committee on Animal Nutrition (SCAN), the use of *Bacillus licheniformis* NCTC 13123 in feedstuffs for pigs is considered unsafe due to the risk of dissemination of genes that confer resistance to clinically important

antimicrobials via the food chain (European Commission 2000). In reaching this decision, SCAN recognised that the magnitude of the risk could not currently be quantified, but is probably low. However given the continuing widespread use of macrolide antimicrobials in pig production, and the selective pressure this provides, SCAN decided to adopt a precautionary approach consistent with its previously expressed Opinion on antimicrobial resistance genes in microbial products.

### VI-I-ii- *B. licheniformis* as a probiotic

*B. licheniformis* is included in a product classified as a probiotic (ZooLac®, ChemVet DK AS, Denmark). ZooLac® is sometimes given to animals for the dietary management of digestion disorders. The total usage of ZooLac® in Norway in 2005 was 776 litres paste (670 litres in 2004). We are not aware of any antimicrobial resistance property of the *B. licheniformis* used in Zoolac.

## VI-II- Bacitracin

Bacitracin is a hexapeptide antibiotic, with a substituted thiazolidine nucleus, produced by some strains of *B. licheniformis* and *Bacillus subtilis* (Azevedo *et al.* 1993). Six compounds have been detected, A, B, C, D, E, and F. Compound A represents about 70% of the complex. Bacitracin is a yellow-white powder, soluble in water and alcohol and insoluble in ether, chloroform, benzene, and acetone. Bacitracin is not absorbed in the gastrointestinal tractus and is degraded in the intestinal lumen. In infants, it is sometimes administered intramuscularly for the treatment of pneumonias. This formulation is sold under the brand name Baciim®. When applied locally (to the skin/mucous membrane), bacitracin is weakly absorbed. In Norway, bacitracin was formerly used as growth promoter in animal husbandry. For many years, bacitracin has been used in some topical preparations for human and veterinary medicine.

### VI-II-i- Mode of action

Bacitracin is mainly active against Gram-positive bacteria, although many differences in susceptibility exist among the bacterial species (O'Grady and Greenwood 1997). The best characterized bactericidal effect of bacitracin is the inhibition of peptidoglycan biosynthesis (Storm 1974), but it may also interfere with additional cellular processes (Butaye *et al.* 2003).

### VI-II-ii- Bacitracin resistance among various bacterial species

Despite its clinical importance, little is known about the mechanisms by which bacteria other than *B. licheniformis* acquire resistance to this antibiotic (Butaye *et al.* 2003;Harel *et al.* 1999).
Nevertheless, resistance mechanisms have been described for a few bacterial species including *E. coli*, *B. subtilis*, *Streptococcus mutans* and *Enterococcus faecalis*.

### VI-II-iii- Resistance to bacitracin in *B. licheniformis*

For self-protection, *B. licheniformis* producer-strains are equipped with resistance mechanisms enabling them to combat the effects of their own metabolic products. An ABC transporter system (ATP-binding cassette) is considered to be the mechanism behind bacitracin resistance in *B. licheniformis* (Podlesek *et al.* 1995).

The transporter system, which has been proposed to mediate active efflux of bacitracin (Neumuller *et al.* 2001;Podlesek *et al.* 1995), is composed of two membrane-proteins, BcrB and BcrC, and two ATP-binding subunits, BcrA. In *B. licheniformis*, the *bcrABC* genes are localized about 3 kb downstream from the bacitracin biosynthesis operon *bacABC* (Neumuller *et al.* 2001).

### VI-II-iv- Known bacitracin resistance mechanisms in other bacterial species

In *E. coli*, the *bacA* gene is involved in the synthesis of a lipid-carrier protein (undecaprenyl phosphate, $C_{55}$-P) essential for peptidoglycan synthesis (El Ghachi *et al.* 2005). Bacitracin is known to block the synthesis and recycling of undecaprenyl phosphate by forming a complex with the precursor $C_{55}$-PP. Over-expression of *bacA* is probably associated with a bacitracin-resistant phenotype in *E. coli* (Cain *et al.* 1993). A different bacitracin resistance gene, $bcrC_{ec}$, encoding a homologue of the resistance gene in *B. licheniformis*, has also been described in *E. coli* (Harel *et al.* 1999).
In *S. aureus* and *S. pneumoniae,* genes homologous to the *bacA* gene have been found. It is unclear whether these genes play a role in acquired bacitracin resistance since they seem to be present in a wide variety of bacterial species, including bacitracin-susceptible ones. These genes might be related to the natural susceptibility level of these bacteria to bacitracin (Butaye *et al.* 2003).
In *B. subtilis*, the bacitracin biosynthesis gene cluster from *B. licheniformis,* including the genes for self-protection, has been experimentally integrated into the chromosome. Synthesis of bacitracin was achieved at high level, as well as expression of the associated bacitracin self-resistance genes (Eppelmann *et al.* 2001).
*Streptococcus mutans* is naturally resistant to bacitracin. RGP (rhamnose-glucose polysaccharide)-synthesis has been shown to be related to bacitracin resistance in *S. mutans* through an unknown mechanism (Tsuda *et al.* 2002).
Studies on bacitracin susceptibility and resistance among enterococci are limited, and a breakpoint for classification of resistant and susceptible isolates has not been determined. Detection and characterization of acquired genes encoding high-level bacitracin resistance in *Enterococcoccus faecalis* has been reported (Manson *et al.* 2004). The genes responsible for this resistance encode an ABC transporter system with some homology to the ABC transporter system present in *B. licheniformis*.

### VI-II-v- Unknown mechanisms

In several bacterial genera, bacitracin resistance has been detected phenotypically, however the mechanisms of resistance remain unclear (Manson *et al.* 2004). Examples of such species are *Clostridium perfringens* (Devriese et al. 1993) and *Streptococcus pyogenes* (Mihaila-Amrouche *et al.* 2004).

### VI-II-vi- Resistance to antimicrobial agents in *B. licheniformis*

Resistance to erythromycin and related antimicrobials is prevalent, but not universal, amongst strains of B*. licheniformis* (Docherty *et al.* 1981). This is also the case for strains deposited in culture collections before 1950, and thus before the widespread use of antimicrobials in human and veterinary medicine.
Resistance against erythromycin in *B. licheniformis* is associated with the *ermK* or *ermD* gene (Docherty *et al.* 1981;Kwak *et al.* 1991). The *B. licheniformis* producer strain used by Alpharma is reported to be resistant to bacitracin, sulfonamides and

erythromycin, although the method used for susceptibility testing is not in accordance to with standardized methodology. Resistance to erythromycin in this strain is mediated by the *ermD* gene. The presence of the *ermD* gene was detected by the use of hybridization with a specific probe (reported during meeting with representatives from Alpharma; 01.02.06). We are not aware that *ermD* gene is transferable from *B. licheniformis* to other bacteria.
The mechanism behind bacitracin resistance in *B. licheniformis* is due to the presence of a transporter system (ATP-binding cassette), as described above in section VI-III-iii.
Resistance to sulfonamides is mediated by an unknown mechanism. The majority of the various *Bacillus* species are reported to be intrinsically resistant against sulfonamides (2005).

### VI-II-vii- Occurrence of bacitracin resistance in Norway

The Norwegian monitoring programme for antimicrobial resistance in bacteria from feed, food and animals (NORM-VET) has investigated resistance to bacitracin in *E. faecalis* and *E. faecium* between 2000-2004 (NORM-NORM-VET 2000;NORM-NORM-VET 2001;NORM-NORM-VET 2002;NORM-NORM-VET 2003;NORM-NORMVET 2004). Up to 50% of the investigated isolates have been reported as resistant against bacitracin. However, the determination of the *in vitro* activity of bacitracin has been bedevilled by a lack of standardized reference methodology, a lack of agreement on interpretive criteria such as breakpoints, and by lack of any great interest in this antibiotic, particularly in human medicine.
Resistance to bacitracin in human pathogens is not investigated in the Norwegian monitoring programme for antimicrobial resistance (NORM). However, reduced susceptibility to bacitracin among *S. pyogenes*, and *S. aureus* isolates of human origin has been recognized in Norway (Arne Høiby, Norwegian Institute of Public Health, personal communication).

## VI-III- Possible dissemination of antimicrobial resistance genes from the *B. licheniformis* producer strain to environmental bacteria

The *B. licheniformis* 20443 strain used by Alpharma to produce bacitracin is highly resistant to bacitracin and sulfonamide, and to a lesser degree erythromycin. The proposed composting of waste from Alpharma's bacitracin production implies that live bacteria and naked DNA present in the waste will come into close contact with environmental bacteria during the composting process. Consequently, the possibility exists that bacitracin, sulfonamide and erythromycin resistance genes can be transferred to the endogenous population of bacilli and other soil bacteria (e.g. enterococci).
Bacteria use three major mechanisms for horizontal gene transfer: conjugation, transduction and natural genetic transformation. Conjugation and transduction are common among bacteria, because they depend on ubiquitous elements such as plasmids, transposons and bacteriophages. Conjugation and transduction require that donor bacteria are alive, whereas horizontal gene transfer by natural transformation depends on naked DNA released from dead donor bacteria. More than 50 different bacterial species have so far been shown to be competent for natural genetic transformation. Among these are the soil bacterium *B.subtilis* and some isolates of *B.licheniformis* (Lorenz and Wackernagel 1994;Rey *et al.*

2004;Spizizen and Prestidge 1969). Although competence for natural transformation has never been demonstrated in *Bacillus cereus* or *Bacillus anthracis* under laboratory conditions, their genome sequences contain homologues of *B. subtilis* competence genes, indicating that natural genetic transformation actually occurs in these bacteria under natural conditions (Vilas-Boas *et al.* 2002).

# VII- Exposure assessment

## VII-I- *B. licheniformis*

### VI-I-i- Ecology

Due to the resistance of their endospores to environmental stresses, as well as their long-term survival under adverse conditions, *B. licheniformis* is ubiquitous and can be isolated from a wide variety of sources. Hence, the occurrence of *B. licheniformis* in a certain environment is not necessarily an indication of habitat and it is generally accepted that the primary habitat is the soil. In the soil environment *B. licheniformis* become metabolically active when suitable substrates for growth are available, and presumably form spores when nutrients are exhausted. This is a strategy used by other microbes in the soil habitat, including the filamentous fungi and the actinomycetes, which also form resting structures (spores), and produce antibiotics in association with their sporulation process.

Since most *Bacillus* species can effectively degrade a series of biopolymers (proteins, starch, pectin, etc.), they are assumed to play a significant role in the biological cycles of carbon and nitrogen.
From soil, by direct contact, or by air-borne dust, *Bacillus* spores can contaminate just about anything that is not maintained in a sterile environment. They may also play a biodegradative role in whatever they contaminate, and thereby they may be agents of unwanted decomposition and decay. Several *Bacillus* species are especially important as food spoilage organisms (e.g. *B. cereus*).

The bacterium is likely to enter the human and animal digestive system many times a day (U.S.Environmental Protection Agency 1997). Data regarding its ability to survive in the gastrointestinal tract are sparse; however, it is highly likely that the spores will pass through without causing harm. Outside the gastrointestinal tract, the bacterium can be a temporary colonizer of skin.

### VII-I-ii-Prevalence of *B. licheniformis* in compost

Various investigations have shown that many different types of thermophilic bacteria are involved in decomposing protein and other readily degradable organic matter (Ichida *et al.* 2001;Wehner *et al.* 2002;Zhang *et al.* 2002). They appear to be responsible for the intense activity characteristic of the first few days, when temperatures reach 65° to 70 °C. Thermophilic species belonging to the genus *Bacillus* are very common in both thermogenic, post-thermogenic and final compost samples, e.g. *Bacillus thermodenitrificans*, *B. licheniformis*, *B. macerans* and *B. stearothermophilus.* Viable cell numbers range from 1.5 to 150 × $10^6$ colony forming units per gram compost (wet weight), with the highest counts being from samples with temperatures of 70 °C and 55 °C. In one study (Zhang *et al.* 2002), *B. thermodenitrificans* was the dominant isolate (representing more than 50% of isolates from total plate counts) in 7 of the 11 individual compost total plate count samples

between 30 ℃ to 73 ℃, and accounted for 68.9% of all isolates. *B. licheniformis* accounted for 7.2% of all isolates and was the dominant isolate in one sample.

### VII-I-iii- *B. licheniformis* 20443 in compost produced from waste

The waste product from the bacitracin production, which is designated "slurry" and is mixed with bark chips and lime and subjected to composting, contains $10^4$-$10^5$ total colony counts per gram. Due to methodological limitations, the fraction of *B. licheniformis* 20443 cells was not determined, but the slurry may contain between $10^2$-$10^3$/g living 20443 cells. Viable spore counts in the slurry were less than 10 spores /ml. There was a lower total cell count in composted slurry than in composted sludge and composted household waste. However, the composted slurry was older than the other compost types. Numbers of viable spores were about the same. The dominant *Bacillus* spp. present in soil and compost were tentatively assigned to the *Bacillus cereus* group (information and data delivered by Alpharma AS and Lindum AS).

### VII-II- Concentration of bacitracin in compost and soil

According to the most recent estimates using LC-MS analysis, composted waste from Alpharma contains 345 ppm bacitracin after 3 days, 31 ppm after 7 days and about 3 ppm after 25 days. Based on "spiking" of compost with a known amount of bacitracin and subsequent extraction by a mixture of methanol, water, and acetic acid (90:9:1), recovery of bacitracin was estimated to be in the range of 50-70%.
Annual waste production is about 1400 metric tons (30 % dry weight). This may amount to 1500-2000 metric tons (dry weight) of finished compost including bulking agents (due to the liquid character of the waste, large quantities of bark or suitable bulking material have to be added). A maximum of 40 metric tons compost per hectare of agricultural land is allowed for quality class I compost products over a 10-year period. Thus, annual application of 1600 metric tons of compost to agricultural soil requires 400 hectares of available farmland. As the compost contained 3 mg bacitracin per kg after 25 days, if a content of 1 mg per kg at the time of application is assumed this corresponds to 40 grams per hectare or about 10-15 µg per kg soil (about 100 µg per litre soil water).

### VII-III- Exposure to antimicrobial resistance genes

Clearly, the amount of bacitracin present during the first week of compost fermentation (see paragraph above) is sufficient to exert a selection pressure on the susceptible members of the bacterial population. Consequently, conditions that promote dissemination of bacitracin resistance genes to natural strains will prevail for a considerable period of time. Presumably, significant amounts of free DNA from *B. licheniformis* 20443 will be present in the waste material used for compost production (see data gaps section). This DNA may be taken up by naturally competent bacilli present during the first week of fermentation. Therefore, conditions promoting the dissemination of bacitracin resistance genes seem to be present during the first week of compost fermentation.
However, in the absence of a corresponding selection pressure, dissemination of sulfonamide and erythromycin resistance genes from the *B. licheniformis* producer strain is unlikely to exceed background level, i.e. the level of naturally occurring gene transfer in soil.
According to a study from Danish Research Centre for Organic Farming (www.darcof.dk/enews/june04/gmo.html) composting of transgenic plant material has

indicated that DNA is degraded much faster than structural plant material, and DNA could no longer be detected after 14 days when the temperature peaked at 58 $^{o}$C. At higher temperatures DNA disappeared after 6 days. In soil, transgenic DNA could still be detected after 77 days (Magid *et al.* 2004). These experiments showed that composting of GM plant residues greatly increases the rate of degradation of transgenic DNA compared to the rate for plant residues left in the soil. However, despite these observations, it cannot be concluded that horizontal gene transfer cannot take place.
In general, the presence and fate of antimicrobial resistance genes in compost has rarely been assessed. In a compost preparation produced from a horse-bedding straw-deep-litter chicken manure mixture supplemented with gypsum, background resistance of the compost microflora to selective agents (kanamycin, novobiocin, tetracycline, thiostrepton, and NaCl) was demonstrated to be extremely varied (Amner *et al.* 1988). Real-time PCR assays may provide rapid, quantitative cultivation-independent measurements of resistance genes. The utility of real-time PCR assays has recently been demonstrated by quantifying three tetracycline resistance (*tet*) gene groups present in bovine and swine manures, composts of swine manure, lagoons of hog house effluent, and samples from an Ekokan upflow biofilter system treating hog house effluent (Yu *et al.* 2005). The bovine manures were found to contain fewer copies of all three groups of *tet* genes than the swine manures. Composting of swine manures substantially reduced *tet* gene abundance (by up to 6 logs), while lagoon storage or the upflow biofilter had little effect on *tet* gene abundance. In cases where the antimicrobials are rapidly degraded, these results suggest that composting may have a substantial impact on the persistence and dissemination of resistance genes that have no other selective advantage.

### VII-IV- Use of bacitracin in Norway

Two preparations containing bacitracin are registered for use in Norway (Bacimycin® powder and Bacimycin® ointment).
In Norway, the usage in 2005 was 400 kg powder and 1800 kg ointment.
This corresponds to 1000 million IU (International Unit) bacitracin (Hege Salvesen Blix, personal communication). The same preparation is used in both human and veterinary medicine. Bacitracin has not been used as a feed additive for domestic animals in Norway since 1997(NORM-NORMVET 2004).
According to the Norwegian monitoring programme for antimicrobial resistance in bacteria from humans (NORM), a marked increase in isolation frequencies of fusidic acid resistant *S. aureus* associated with *impetigo bullosa* among children has been recognized. The Norwegian monitoring programme for antimicrobial resistance in bacteria from feed, food and animals (NORM-NORM-VET 2000;NORM-NORM-VET 2001;NORM-NORM-VET 2002;NORM-NORM-VET 2003;NORM-NORMVET 2004) has registered the same tendency regarding the occurrence of fusidic acid resistant staphylococci isolated from animals.Therefore, bacitracin has been suggested as one of the drugs of choice for treatment of impetigo in Norway.

## VIII- Risk characterization

### VIII-I- Survival of *B. licheniformis* 20443 bacitracin producer strain

Experiments conducted by Alpharma indicate that the production strain accounted for less than 1% of all compost isolates. Most isolates belonging to the genus *Bacillus,*

were assigned to the *B. cereus* group. If this is correct, the production strain may differ substantially from the range of phenotypes exhibited within the species and apparently displays a selective disadvantage compared to the original strain when reintroduced to soil.

### VIII-II- Bacitracin

In the spiking experiment, bacitracin was allowed to stay in contact with the solid phase of the compost for only 20 min before extraction. In our opinion, this period may have been too short for bacitracin to penetrate into the micropores in the solid-phase material, and for bactracin to form stable non-covalent complexes with the solid phase. It is therefore possible that the estimated recovery is too high and not applicable to the situation where bacitracin has stayed in contact with the solid-phase for a longer period. However, low recovery may indicate less bioavailability and reduced selection pressure. Regardless of recovery, sufficient residual bacitracin is present in the initial phase of the composting process to exert a selection pressure that promotes dissemination of bacitracin resistance genes. Residual bacitracin in the final product is low and less likely, if at all, to promote selective advantage to resistant soil or compost bacteria.

### VIII-III- Propagation of antimicrobial resistance from the *B. licheniformis* 20443 bacitracin producer strain

Undoubtedly antimicrobial resistance genes will be transferred from the *B. licheniformis* bacitracin producer strain to members of the genus *Bacillus* and other bacteria present in the compost environment, if compost containing the producer strain is mixed with soil. Studies on agricultural soil treated with animal waste, and laboratory experiments carried out using soil microcosms, support this view, but the efficiency of the gene transfer processes in soil is believed to be relatively low (Graham and Istock 1979;Jensen *et al.* 2001;Lorenz and Wackernagel 1994). Consequently, without a selection pressure promoting the survival and propagation of soil bacteria that have acquired antimicrobial resistance genes from the *B. licheniformis* producer strain, the number of transformants present in the enriched soil environment will be very small. In contrast, in environments where bacitracin, sulfonamide, or erythromycin is present at concentrations affecting bacterial growth, bacteria acquiring antimicrobial resistance by incorporating resistance determinants from the *B. licheniformis* producer strain will have a selective advantage and potentially reach high numbers.
As resistance to sulfonamide, and erythromycin is common among *B licheniformis* (Docherty *et al.* 1981) and other soil bacteria (D'Costa *et al.* 2006), the presence of a low number of *B. licheniformis* 20443 cells carrying these determinants is not considered to present a significant risk to human health or the environment.

## IX- Data gaps

During the preparation of this assessment, the following data gaps were identified:

- Insufficient documentation on the number of surviving *Bacillus licheniformis* 20443 cells present in the compost after fermentation.
- No information on the amount of free DNA from *Bacillus licheniformis* 20443 present in the waste material used for compost production.

- Lack of use of simple molecular typing tools (like Random Amplification of Polymorphic DNA; RAPD) to differentiate between the *B. licheniformis* production strain 20443 and other *B. licheniformis* present in compost/soil.
- Lack of information on the occurrence and presence of pathogenic bacteria (e.g. enterococci) in the composting system.
- The extent to which the resistance genes in *B. licheniformis* are transferable and can mediate phenotypically-expressed resistance in other bacterial species, like human and animal pathogens, is not documented.
- An absence of a standardized method to determine bactericidal and bacteriostatic (Minimum Inhibitory Concentration, MIC) values for bacitracin.

# X- Answers to the questions

**1- Assessment of risk in relation to residual content of bacitracin in the finished product.**
**Will the residual content of bacitracin in the proposed compost pose any risk to human health or the environment?**

According to the measurements conducted and presented by Alpharma, after the production waste has been mixed with bark chips and lime and composted for 25 days, the concentration of bacitracin is about 3 ppm or less. It is highly unlikely that bacitracin at this concentration will pose a significant risk to human health or have adverse toxicological effects. However the antibiotic may exert a selection pressure on the bacterial community in the compost (see below), and thus a possible long-term effect cannot be excluded.

**2- Assessment of risk in relation to dissemination of the production strain**

**a- Assess the risks of disseminating *B. lichenformis* 20443 to the environment, and whether such dissemination could pose a threat to human health or the environment?**
It is likely that *B. licheniformis* 20443, which is the laboratory mutant strain used by Alpharma to produce bacitracin, has lost some of its ability to compete with bacteria found in the environment. The 20443 strain does not form spores during the production process, and due to the low pH (pH 4.0) relatively few living bacteria ($10^2$-$10^3$ cfu/g) are present in the waste used for composting. Furthermore, studies carried out by Alpharma suggest further reductions in strain 20443 in the waste product during the composting process. However, more accurate data are required to confirm these findings. Considering the low number of producer bacteria remaining in the finished product, the probability is low that it will disseminate from the compost. Consequently, because *B. licheniformis* appears to be essentially non-pathogenic to healthy people, and very uncommon as an opportunistic pathogen in humans, the bacterium constitutes a minimal risk to human health or the environment.
The situation will differ if sufficient bacitracin is present in the compost to inhibit growth of a significant fraction of the natural population. This scenario is unlikely, however, as a concentration of about 3 ppm bacitracin will probably be too low to exert sufficient effective selection pressure to enable the bacitracin-resistant

producer strain to out-compete other bacteria. Additionally, only Gram-positive bacteria are susceptible to bacitracin.

**b- Assess the risks of dissemination of antibiotic resistance genes from the compost.**

The waste product designated “slurry”, which is mixed with bark chips and lime and subjected to composting, contains relatively high amounts of bacitracin. In addition this slurry may contain $10^2$-$10^3$ living *B. licheniformis* 20443 cells per gram plus unknown amounts of free DNA released from dead and disintegrated producer bacteria. Consequently, it is likely that during the first week of composting, selection pressure will favour the transfer of bacitracin-resistance genes to different strains and species of *Bacillus* and possibly other species growing in the compost. It is not possible to predict the extent of such lateral gene transfer, but it is reasonable to assume that under the special conditions prevailing early in the composting process, increased dissemination of the bacitracin-resistance genes to the environment may take place relative to background level. However, this is considered to represent a low risk to human health and the environment.
The abundance of resistance genes in the finished compost will probably be close to the background level and application of the final product as a soil amendment will not contribute to increased dissemination of bacitracin-resistance. However, this is considered to represent a low risk to human health and the environment.

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